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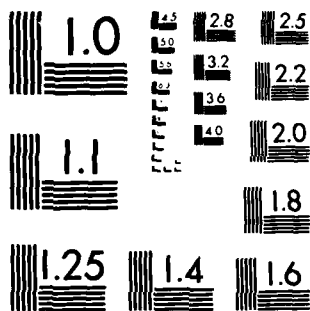
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An Investigation of the Memory Response of the Local
Immune System to Shigella Antigens

Annual Report

David F. Keren, M.D.

January 1982

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number) Rabbits were immunized orally with 3 doses of live noninvasive, Shigella flexneri strain 2457-0. Sixty days after the third oral dose a chronically isolated ileal loop was created surgically in each rabbit. The local IgA and IgG anti-shigella responses in secretions and sera were followed with a sensitive enzyme-linked immunosorbent assay. A significant local IgA memory response was found in the secretions of the orally-primed rabbits. Parenteral		

immunization alone results in erratic and weak local IgA production. Further, with a dosage schedule was that achieved serum IgG activity to shigella antigens, parenteral immunization was not able to prime the rabbits for local, intestinal IgA memory response. In other studies, it was found that erythromycin interfered with development of IgA memory responses and adjuvant (DEAE-dextran) had no significant effect on the primary local IgA response to orally-administered Shigella flexneri. The effect of this proposed adjuvant on the IgA memory response remains to be investigated the present studies demonstrate that a local IgA memory response to Shigella flexneri can be elicited by oral priming with a live, noninvasive strain. Further, parenteral vaccination was ineffective in priming for a mucosal IgA memory response.



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SUMMARY

In the present studies, we have used our chronically isolated ileal loop model in rabbits as a probe to study the variables involved in eliciting a mucosal IgA memory response to shigella antigens. Our previous studies have documented that a local IgA memory response to shigella antigens could be elicited by priming rabbits with three oral, weekly doses of 10^{10} live Shigella X16. No such local IgA memory response in intestinal secretions was found when heat-killed shigella were given in the same dosage and route. In the present studies we examined whether this was due to the fact that Shigella X16 is capable of invading the surface epithelium. In the first group of experiments performed this year we found that oral priming with three weekly doses of 10^{10} live, non-invasive Shigella flexneri strain 2457-0 was capable of priming for as vigorous a local IgA memory response as the live locally invasive Shigella X16. Therefore invasion per se is not a requisite for an oral vaccine strain to elicit a local IgA memory response.

As most traditional methods of immunization involve parenteral vaccines, we have examined the effect of giving a primary parenteral immunization on the local immune response of the intestine with our chronically isolated ileal loops as the probe. Secretions from isolated ileal loops of animals given a primary parenteral immunization with shigella antigens showed that little local IgA is produced, while good serum titers of IgG were elicited. It was noted, however, that more locally produced IgG (in parallel with serum IgG) is found in these animals than in animals that are given shigella only by oral immunization.

A second group of animals was primed parenterally, allowed to rest for one week, and then challenged with a single oral dose of live locally invasive shigella. This was designed to test the presence or absence of a local memory response to this intestinal challenge. Again, only a little local IgA was produced and no booster effect of the IgG was seen either locally or in the serum following this single oral challenge dose. Therefore, it seems clear that in a previously unimmunized animal parenteral priming with shigella antigens produces only a weak local IgA response and no priming for a local memory IgA response.

Our preliminary investigation of DEAE-dextran as a mucosal adjuvant has shown no enhancement of the primary local IgA response by this agent when used with the oral heat-killed shigella. The effect of this adjuvant on subsequent mucosal memory response will be determined in later experiments.

Lastly, we record a peculiar effect of the antibiotic erythromycin on the local IgA memory response of orally-primed rabbits. When rabbits were treated with erythromycin at the time of their challenge dose, no local IgA memory response could be demonstrated. The mechanism of action of this effect is unclear at the present time. This effect could be of importance, however, in any vaccine program using live, attenuated strains.

FORWARD

During the course of this work, the author was greatly assisted by Patricia Scott, Diana Lauer, Pamela Porter, Scott Kern, Arthur Rosner, and Roderick McDonald. In addition, the excellent Laboratory Animal Medicine Department at the University of Michigan continues to provide superb care for our animals. Their help is deeply appreciated. The excellent assistance of MaryAnn Byrnes in preparing this and other manuscripts related to this project is appreciated.

In conducting the research described in this report, the investigator adhered to the "Guide for Care and Use of Laboratory Animals" prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication # (NIH) 78-23, 1978).

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INTRODUCTION

This second annual report includes work completed from 1 February, 1981 to the present (31 January, 1982).

In our original proposal, we hypothesized that the chronically isolated ileal loop model in rabbits could be used as a probe of the local IgA response to *Shigella flexneri*. The feasibility of this approach evolved from studies of antigen stimulation of mucosal immunity and lymphocyte trafficking in the bowel. In the bowel, antigen is taken up by specialized epithelial cells that cover the dome regions of Peyer's patches (1,2). This antigen then stimulates IgA precursor B lymphocytes and regulatory T lymphocytes in the Peyer's patches (3-6). After local antigen stimulation, these lymphocytes migrate to the systemic circulation, and eventually travel back to the mucosal surface of the gastrointestinal tract as well as other mucosal surfaces (bronchial mucosa, mammary gland, etc.) (7-9). The importance of looking at intestinal secretions to follow mucosal immunity was emphasized by La Broody et al's recent demonstration of a poor correlation between salivary and jejunal antigen-specific IgA (10).

Last year, in our first annual report we presented data that the chronically isolated ileal loops can serve as probes for following the local immune response to orally - administered antigens. Further, by using this chronically isolated ileal loop probe, we presented preliminary information that a strong local IgA memory response was produced in intestinal secretions following oral immunization with the *S. flexneri* - *Escherichia coli* (*Shigella* X16) hybrid strain. This was the first direct demonstration in intestinal secretions of a mucosal IgA memory response to *S. flexneri* given orally. Local IgA memory was only elicited when the *Shigella* X16 were given as a live preparation, not when the original vaccine was heat-killed (11).

The finding that a local IgA memory response was elicited by oral immunization with live but not killed *Shigella* X16 left several questions to be answered. Since *Shigella* X16 is a locally invasive hybrid, we needed to determine whether invasion per se was needed to elicit IgA memory. In this year's work we report results with noninvasive *S. flexneri* strain 2457-0.

Further, as most traditional means of immunization involve parenteral vaccines, we wanted to determine with our chronically isolated ileal loop model, if a vigorous local IgA response could be elicited by such peripheral stimulation. This year's report details the studies which demonstrate the relatively poor local immune response elicited by parenteral immunization.

While performing our investigations, occasional rabbits would be treated for respiratory tract infections with antibiotics. When rabbits were being treated with antibiotics at the time of challenge, the local

IgA memory response was affected. Further studies have been performed to confirm this effect which could have practical importance in a vaccine program.

Lastly, work with a potential mucosal adjuvant, SEAC-Phytran (12), has begun. Preliminary data shows no effect of this adjuvant in the primary local IgA response to Shigella X16.

METHODS

Preparation of Chronically Isolated Ileal Loops

The surgical creation of ileal Thiry-Vella loops in rabbits has been described in detail previously (13). In brief, while 3-4 Kg New Zealand White rabbits are anesthetized with Rompum and Ketamine, a midline abdominal incision is made and the terminal ileum is identified. A 20 cm segment of ileum containing a grossly identifiable Peyer's patch is isolated with its vascular supply intact. Silastic tubing (Dow-Corning) is sewn into each end of the isolated segment. This tubing is brought out through the midline incision and tunneled subcutaneously to the neck where it is exteriorized and secured. Intestinal continuity is restored by an end-to-end anastomosis and the midline incision is closed in two layers.

Each day about 2-4 ml of secretions and mucus that collect in the ileal loops are expelled by injecting 20 cc of air into one of the silastic tubes. The slightly opaque, colorless fluid and mucus expelled from the tubing is studied for specific immunoglobulin content. A subsequent flush with 20 cc of sterile saline helps to remove adherent mucus. This saline is then removed by repeated gentle flushes of air. With proper daily care, 80-90% of rabbits can complete experiments lasting 1-2 months.

Enzyme-Linked Immunosorbent Assay (ELISA).

Briefly, microtiter wells are coated with a solution containing shigella lipopolysaccharide (Westphal preparation). Immediately prior to testing serum samples or loop secretions, the antigen solution is removed and wells are washed with a phosphate buffer containing Tween 20 (PT). The fluid to be assayed is diluted in the PT buffer and incubated in the coated wells and in uncoated wells (to control for nonspecific adsorption) for four hours on a horizontal rotary shaker. The plates are washed with PT and incubated with either alkaline phosphatase-conjugated goat anti-rabbit IgG or IgA overnight on the shaker. Following another PT wash, substrate reaction is carried out with nitrophenyl phosphate in carbonate buffer. The OD 405 nm of the substrate reaction is determined using a Titer Tek microelisa reader. Kinetics of the enzyme-substrate reaction are extrapolated to 100 minutes. The OD 405 nm of uncoated wells are subtracted from the OD 405 nm of coated wells. Specific IgG and IgA standards are processed daily with the unknown fluids as previously described (8).

Table 1. Immunization Schedule

<u>Group</u>	<u>Antigen</u>	<u>Dose</u>	<u>Route</u>	<u>Day(s)⁽¹⁾ Given</u>
I	Live <u>S. flexneri</u> 2457-0	10^{10}	oral ⁽²⁾	0
II	Live <u>S. flexneri</u> 2457-0	10^{10}	oral	0,7,14
III	Live <u>S. flexneri</u> 2457-0	10^{10}	oral	-75,-68,-61,0
IV	Heat-killed <u>Shigella</u> X16	10^8	subcutaneous	0,1
	Heat-killed <u>Shigella</u> X16	10^8	intravenous	4-8,14
V	Heat-killed <u>Shigella</u> X16	10^8	subcutaneous	-21,-20
	Heat-killed <u>Shigella</u> X16	10^8	intravenous	-17 --13,-7
	Live <u>Shigella</u> X16	10^{10}	oral	0
VI	Live <u>Shigella</u> X16 with DEAE-dextran	10^{10}	oral	0
		5 gm/dl	oral	
VII	Live <u>Shigella</u> X16 Erythromycin	10^{10}	oral	-75,-68,-61,0 -2,-1,0,1,2,3

(1) Day of surgical creation of isolated loops =
day -1 for all groups.

(2) Shigella placed in stomach via orogastric tube. Isolated loop not
directly exposed to Shigella.

RESULTS

The local IgA anti-shigella response in isolated loop secretions following a single oral dose of 10^{10} live (noninvasive) *S. flexneri* strain 2457-0 is shown in figure 1. The kinetics of the local anti-shigella response is similar to our previous studies wherein the isolated ileal loops were directly immunized with live *S. flexneri* strain 2457-0 (14). Further there was no significant difference between this response and the response to a single oral dose of 10^{10} live (invasive) *Shigella* X16 (11).

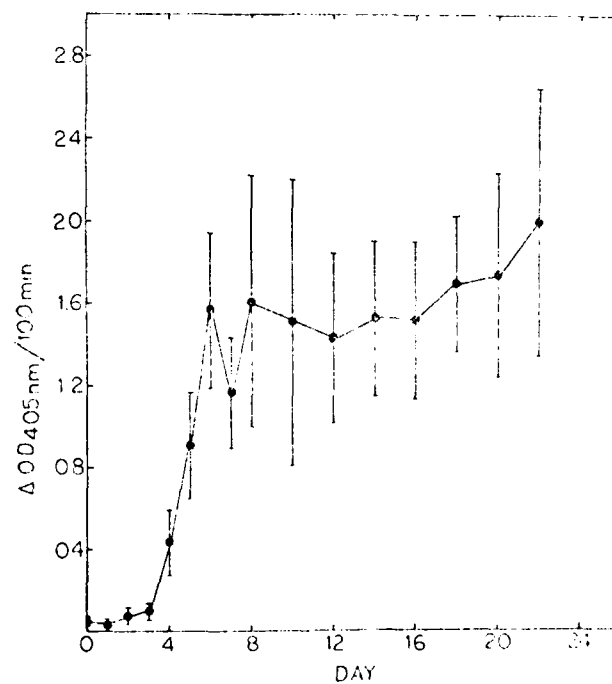


Figure 1. Mean IgA responses to *Shigella* LPS in secretions from rabbits given a single oral dose of 10^{10} noninvasive, live, *Shigella flexneri* strain 2457-0 on day 0. IgA activity \pm S.E. indicated on vertical axis.

Our previous studies using direct immunization of the isolated ileal loop demonstrated that multiple weekly immunizations produced a greater individual IgA response (11). To determine whether this would occur in this system, the Group II animals were given 3 weekly, oral doses of 10^{10} live *Shigella flexneri* strain 2457-0. The IgA anti-shigella activity from the isolated loop secretions of the Group II rabbits is shown in figure 2. No significant difference was found between these values and those of the Group I animals.

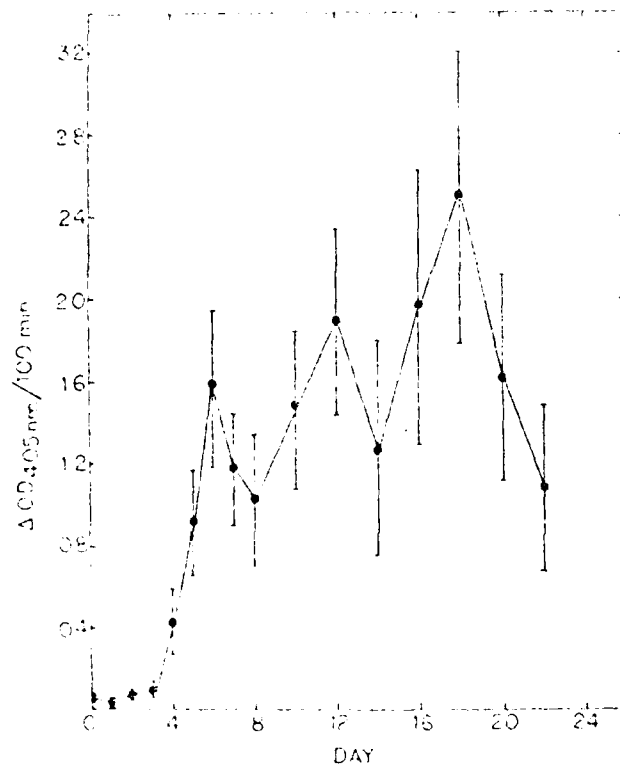


Figure 2. Mean IgA responses to shigella LPS in secretions from rabbits given oral doses of 10^{10} noninvasive, live, *Shigella flexneri* strain 2457-0 on days 0, 7 and 14. IgA activity \pm S.E.M. indicated on vertical axis.

To determine whether a local IgA memory response could be elicited by immunization and challenge with noninvasive *S. flexneri*, the Group III rabbits were primed with three weekly oral doses of 10^{10} live *S. flexneri* 2457-0. Sixty days after the third oral dose of *S. flexneri*, a chronically isolated ileal loop was created and the animals were challenged with a single oral dose of 10^{10} live *S. flexneri* 2457-0. As shown in Table 2, an IgA memory response was found in the orally-primed rabbits. As was true with our studies using locally invasive *Shigella* X1C, a low level of IgA activity against shigella was maintained in many of the animals even after 60 days (11).

TABLE 2

IgA Anti-Shigella in Loop Secretions

Day After Challenge (1)	Not Primed (2)	Primed (3)	Significance (5)
0	.061 ± .017 ⁽⁴⁾	.397 ± .267	N.S.
1	.146 ± .038	.216 ± .109	N.S.
2	.109 ± .039	.276 ± .126	N.S.
3	.176 ± .070	.571 ± .232	< .001
4	.423 ± .153	2.030 ± .400	< .001
5	.894 ± .242	2.434 ± .493	< .001
6	1.580 ± 1.398	2.671 ± .498	N.S.

(1) Day 0 = Day of final challenge

(2) Unprimed Animals given 10^{10} S. flexneri 2457-0 on day 0 (N=16).

(3) Animals primed orally with 10^{10} S. flexneri 2457-0 on Days -75, -68, -61 prior to oral challenge on Day 0 (N=10).

(4) Results expressed as mean O.D. 405 nm/100 min. ± S.E.M.

(5) Significance determined by F-test.

(6) N.S. = not significant.

These findings demonstrate that ingestion is not necessary for the vaccine to elicit local IgA memory after oral immunization. Our original demonstration that live *Shigella* X16 were able to prime for a local IgA memory response while heat-killed *Shigella* X16 could not, must relate to other factors. It needs to be determined whether the live bacteria, because of their ability to multiply in the gastro-intestinal tract, provide a much higher dose of antigen. This will be examined by increasing logarithmically the dose of heat-killed shigella given orally to prime the animals. Alternatively, it may be that the animals given heat-killed shigella in our previous studies (11) had the ability to give a local memory response, but that our challenge with killed shigella was inadequate. To address this issue future studies will challenge these groups with live *Shigella* X16.

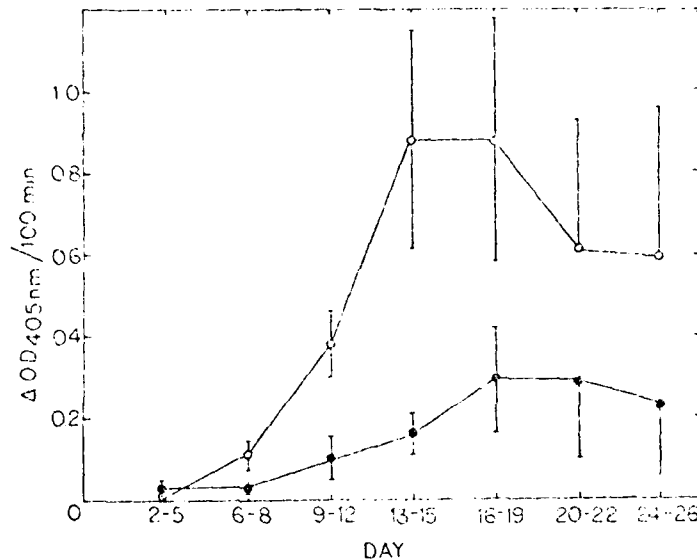


Figure 3. Mean serum IgG (o) and IgA (●) activity to shigella LPS in rabbits given 10^8 heat-killed *Shigella* X16 subcutaneously on days 0 and 1, and intravenously on days 4-8 and 14. (Group IV).

As parenteral administration has long been used to stimulate the immune response to infectious agents including shigella, we have examined the effect of this route of immunization on the local IgA activity to *Shigella* X16. The rabbits in Group IV were treated with a regimen of subcutaneous and intravenous injections previously used to elicit strong serum antibody to this bacteria (14) (Table 1).

The isolated ileal loops were created on day 0 (Table 1) and secretions from these loops were studied for their IgG and IgA activity to *Shigella* X16. The serum IgG and IgA activity is depicted in Figure 3. As seen previously, all rabbits developed strong IgG anti-*Shigella* activity, but the IgA anti-*Shigella* activity was variable and weaker than when animals were immunized orally.

In the loop secretions, the IgG activity paralleled the serum activity, but was weaker (figure 4). The local IgA response was extremely variable (both day to day and between rabbits) and weak as compared to local or oral immunization (figure 4).

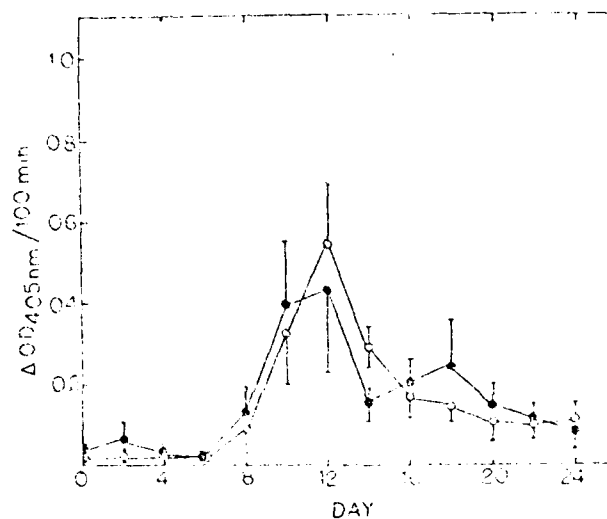


Figure 4. Mean IgG (o) and IgA (c) activity to shigella LPS in secretions from Group IV rabbits.

Despite the relatively weak primary immune response, it is possible that the parenteral immunization primed the animals to give a local IgA memory response to a subsequent single oral challenge with live *Shigella* X16. Therefore, in Group V the rabbits were given the same parenteral regimen as the Group IV rabbits (Table 1).

However, the isolated ileal loops were not created until one week after the last intravenous dose of *Shigella* X16. The serum IgG and IgA activity to shigella were similar to those of the Group IV animals (figure 5).

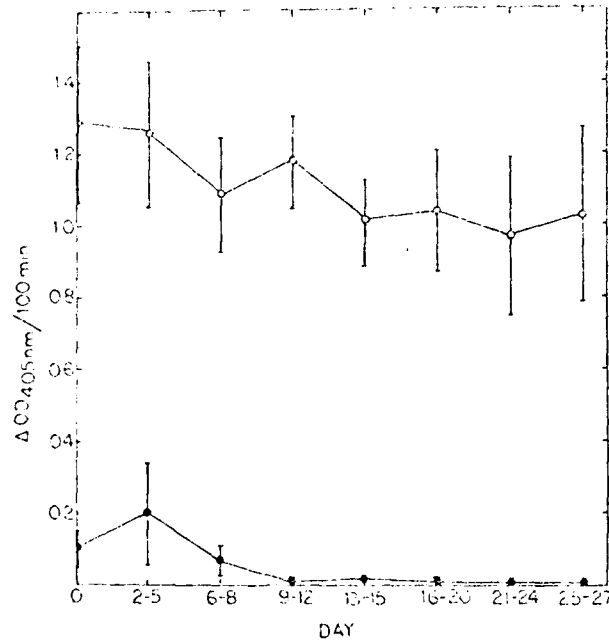


Figure 5. Mean serum IgG (○) and IgA (□) activity to shigella LPS in rabbits given 10^6 heat-killed *Shigella* X16 subcutaneously on days -21, -20, and intravenously on days -17, -13, and -7 followed by a single oral dose on day 0 (Group V).

The local IgA activity after the systemic priming and subsequent single oral challenge with live *Shigella* X16 showed a typical primary local IgA response (figure 6). There was no evidence of an IgA memory response and no evidence of suppression of the local IgA response. The local IgG response, again, paralleled the serum IgG activity with no evidence of enhanced response after the single oral challenge. These data are consistent with our previous findings that parenteral priming did not enhance the local IgA response to shigella subsequently given directly into the isolated ileal loops (14).

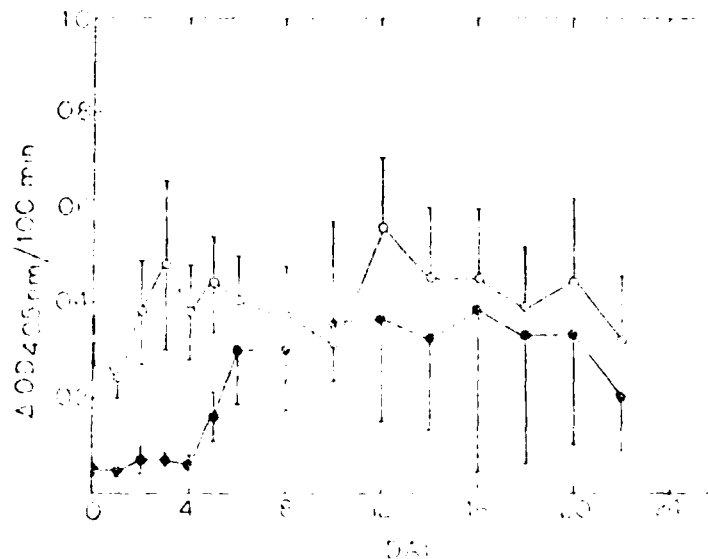


Figure 6. Mean IgG (o) or IgM (e) activity to shigella LPS in serum samples of Group VI animals.

Beh has recently proposed the use of DEAE-dextran as a mucosal adjuvant for oral vaccines to bacteria (12). Therefore, to establish baseline data for future memory response experiments, we looked at the effect of DEAE-dextran on the primary local immune response to shigella. Group VI animals were given DEAE-dextran along with the single oral dose of live shigella X16 (Table I). As shown in Figure 7, a typical primary IgA response to the single oral dose of shigella resulted. There was no significant effect, indeed, the mean IgA response were lower in the DEAE-dextran group, although this was not statistically significant. Histologic studies of the stomach, and small and large intestine revealed no evidence of damage by oral administration of DEAE-dextran at 1, 6, 24 and 48 hours.

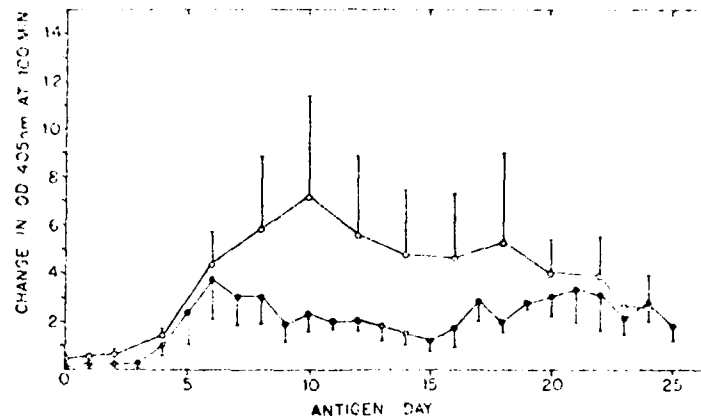


Figure 7. Mean IgA anti-shigella activities in secretions from rabbits fed a single live, oral dose of 10^{10} Shigella X16 on day 0 (○) and from rabbits given BSA-dextran along with the single live, oral dose of 10^{10} Shigella X16 on day 0 (◻).

In early studies, we observed that an occasional rabbit gave poor or no local IgA memory responses following the triple, oral, live Shigella X16 priming regimen. By studying our daily logs, we discovered that these animals had all received antibiotic therapy for respiratory infections at the time of the single oral challenge dose. Whereas, none of the rabbits which displayed local IgA memory responses received such antibiotics at time of oral challenge. To investigate

whether this was a phenomenon due to variation of the outbred nature of our rabbits or to a real effect of the antibiotics, several additional rabbits were given oral priming with live *Shigella* X16. The local IgA anti-shigella activity of this group was extremely poor (figure 8). Whether this is due to a specific suppressive action of the antibiotic on the local immune system or whether it is merely due to the effect of the antibiotic on the live oral bacteria needs to be studied.

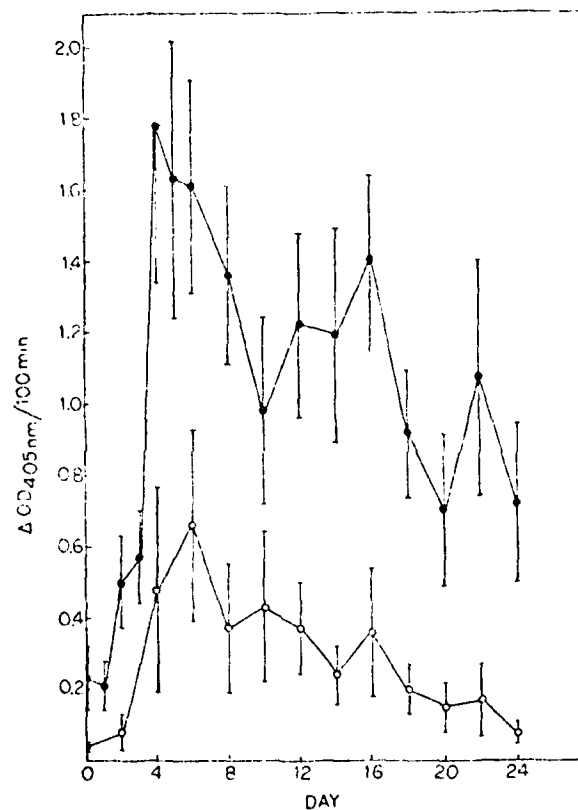


Figure 8. Mean IgA activity to shigella LPS in intestinal secretions from orally primed animals challenged with a single dose of live *Shigella* X16. The group of animals given erythromycin at the time of the challenge dose (○) had a significantly weaker response than animals that did not receive antibiotics (●).

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